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Russula rubricolor sp. nov. from Himalayan forests of Pakistan

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Abstract—A new species, *Russula rubricolor,* was collected in the Himalayan forests of Pakistan. This species is morphologically characterized by the yellowish hymenium, bright red pileus that is convex to flat with central depression and finally infundibuliform and striate towards margin, becoming blackish red with age. The newly reported species is placed in *Russula* subsect. *Maculatinae* based molecular phylogenetic analysis of ITS sequence data and supported by the red pileus color.

Key words—*Cedrus,* phylogeny, *Quercus, Russulaceae*, taxonomy

Introduction

The monophyletic genus *Russula* Pers. (*Russulaceae*) is the largest genus in *Russulales* (Singer 1986, Buyck & Horak 1999, Buyck & al. 2008, Li & al. 2015, Ghosh & al. 2016). Resupinate, discoid, effused-reflexed, clavarioid, pileate, or gasteroid basidiomata can be found in this order (Miller & al. 2006, Kirk & al. 2008, Wang & al. 2009, Li & al. 2018). *Russula* is one of the most species-rich agaric genera in *Russulales,* making species recognition often difficult. The genus is generally easily distinguished from its sister genera *Lactarius* Pers. and *Lactifluus* (Pers.) Roussel in the field by absence of latex and under the microscope by presence of complex agglomerated sphaerocytes and absence of pseudocystidia or lactiferous hyphae (Verbeken 1996). *Russula* subsect. *Maculatinae* (Konrad &Joss.) Romagn. is characterized by the yellow spore print, acrid taste of the flesh, and non-incrusted pileocystidia with contents that turn grey in sulphovanillin (Adamčík & Jančovičová 2012).

Russula is globally distributed and occurs across a wide range of habitats from the tropics to polar ecosystems (Miller & Buyck 2002, Park & al. 2013, Lee & al. 2017). The genus plays an important role in forest ecosystems as its members are ectomycorrhizal symbionts with a wide range of trees including angiosperms and gymnosperms (Richardson 1970; Bills & al. 1986; Villeneuve & al. 1989; Claridge & May 1994; Buyck & al. 1996; Gardes & Bruns 1996; Ahmad & al. 1997; Niazi & al. 2006; Niazi 2008; Razaq & al. 2014; Jabeen & al. 2015, 2016, 2017a,b; Saba & Khalid 2015.

Fungal forays to the Abbottabad and Swat districts were conducted during 2014–16 to inventory the macrofungi of the area. One *Russula* species stood out as different and is introduced here as a new species, based on a detailed morphological description and comparison of its ITS sequences with those available in GenBank.

Materials & methods

Specimens were photographed in field, air dried, described and deposited in the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH). Color codes were given following Munsell soil color charts (Munsell 1975). Potassium hydroxide (KOH 5% aq. w/v) was used to rehydrate dried tissues. Congo red (1% aq. w/v) was used as mounting medium to visualize hyphal walls and contents in microscopic studies. Melzer's reagent and sulphovanillin were used for chemical tests. For scanning electron microscopy, tissue from the hymenium was mounted on a metal stub using double sided adhesive tape and covered with a fine layer of gold in a JEOL ion sputter JFC-1100 and then examined using a JEOL JSM-6360 Scanning Electron Microscope at 20 kV. The notation "basidiospores (*n/m/p*)" indicate that measurements were made from *n* basidiospores of *m* basidiomata and *p* collections. The ornamentation is excluded. Q = l × w ratio; definitions of spore Q values follow Bas (1969).

Genomic DNA was obtained from dried basidiomata using the CTAB procedure (Gardes & Bruns 1993). The primer pair ITS1F (Gardes & Bruns 1993) and ITS4 (White & al. 1990) was used to amplify the ITS region. PCR was performed in a total volume of 25 µl containing 2.5 µl of PCR reaction buffer, 0.6 µl of dNTP mix (2.5 mM), 1 µl of each primer (10 µM), 0.2 µl of *Taq* polymerase, and 0.5 µl of DNA template beginning with a 4 min initial denaturation at 94°C, followed by 34 cycles of denaturation at 94°C for 40s, annealing at 52°C for 60s, at 72°C for 80s, and by a final extension at 72°C for 8 min. The PCR amplicons were purified using Exonuclease I and Shrimp Alkaline Phosphatase enzymes (Werle & al. 1994).

Purified PCR products were sequenced by the Beijing Genomic Institute P.R. in China using the same ITS1F & ITS4 primers. DNA sequences were trimmed, edited, and assembled using Sequencher® 5.41 software. Closely related BLAST-search sequences and published sequences (Adamčík & al. 2016, 2019; Li & al. 2018) were downloaded from GenBank and UNITE to reconstruct the phylogeny. The sequences selected were derived from types (preferred), representatives of same species, and others with complete ITS regions. Environmental sequences closely related to the Pakistani specimens were also included in the final ITS dataset to determine distribution. Ambiguously aligned sequences were excluded after preliminary analysis. Sequences were aligned using MUSCLE v3.6 (Edgar 2004). DNA sequences generated for this study were deposited in GenBank under accession numbers MK142795–MK142798.

A maximum likelihood tree was inferred using Kimura 2-parameter model (K2+G) (Kimura 1980) by best DNA model selection in MEGA 6 (Tamura & al. 2013). A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.4241)]. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved a selection of 121 nucleotide sequences. *Russula sardonia* Fr. (KT933999) and *R. emetica* (Schaeff.) Pers. (JQ888196) were selected as outgroup taxa from *Russula* core clade while others represent *Russula* crown clade (Adamčík & al. 2019). There were a total of 725 positions in the final dataset. One thousand bootstrap iterations were performed with rapid bootstrapping in MEGA 6 for phylogenetic tree construction (Tamura & al. 2013).

Phylogenetic results

BLAST search of the consensus sequence from LAH35070 at NCBI database revealed a 97% identity among LAH35070 and unidentified *Russula* sequences KM576560 from UK and KU914610–KU924613 from China with 100% query cover. It also showed 97% identity with a *Russula* sp. (AF418639) sequence from ectomycorrhizal root tips from Germany, indicating that the sequence from collection LAH35070 (haplotype) separated from all currently available sequences in public databases.

In the phylogram generated by ML analysis of the ITS sequences (Fig. 1), the *Russula rubricolor* sequences clustered in a clade with MG719932 and MG719933 from *R. heilongjiangensis* G.J. Li & R.L. Zhao from China and an unidentified *Russula* sp. (KM57656 from China; KU914610–KU924613 from Germany; and AF418639 from United Kingdom). A species complex comprising *R. globispora* (J. Blum) Bon and *R. dryadicola* R. Fellner & Landa is sister to the *R. rubricolor*/*R. heilongjiangensis* clade. Sequences from the Pakistani collections formed a separate clade with strong bootstrap support of

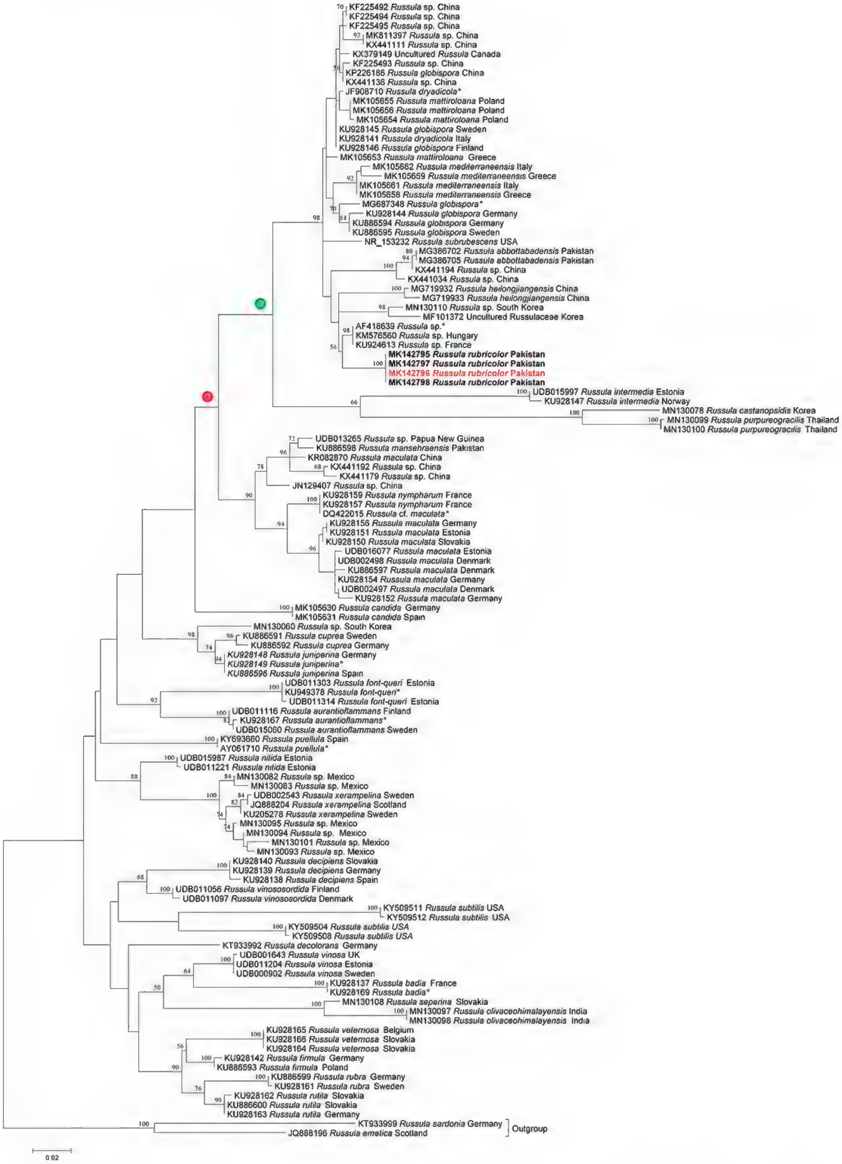
KF225492 *Russula* sp. China
KF225494 *Russula* sp. China
KF225495 *Russula* sp. China
MK811397 *Russula* sp. China
KX441111 *Russula* sp. China
KX379149 Uncultured *Russula* Canada
KF225493 *Russula* sp. China
KP226186 *Russula globispora* China
KX441136 *Russula* sp. China
JF908710 *Russula dryadicola**
MK105655 *Russula mattiroloana* Poland
MK105656 *Russula mattiroloana* Poland
MK105654 *Russula mattiroloana* Poland
KU928145 *Russula globispora* Sweden
KU928141 *Russula dryadicola* Italy
KU928146 *Russula globispora* Finland
MK105653 *Russula mattiroloana* Greece
MK105662 *Russula mediterraneensis* Italy
MK105659 *Russula mediterraneensis* Greece
MK105661 *Russula mediterraneensis* Italy
MK105658 *Russula mediterraneensis* Greece
MG687348 *Russula globispora**
KU928144 *Russula globispora* Germany
KU886594 *Russula globispora* Germany
KU886595 *Russula globispora* Sweden
NR_153232 *Russula subrubescens* USA
MG386702 *Russula abbottabadensis* Pakistan
MG386705 *Russula abbottabadensis* Pakistan
KX441194 *Russula* sp. China
KX441034 *Russula* sp. China
MG719932 *Russula heilongjiangensis* China
MG719933 *Russula heilongjiangensis* China
MN130110 *Russula* sp. South Korea
MF101372 Uncultured Russulaceae Korea
AF418639 *Russula* sp.*
KM576560 *Russula* sp. Hungary
KU924613 *Russula* sp. France
MK142795 *Russula rubricolor* Pakistan
MK142797 *Russula rubricolor* Pakistan
MK142796 *Russula rubricolor* Pakistan
MK142798 *Russula rubricolor* Pakistan
UDB015997 *Russula intermedia* Estonia
KU928147 *Russula intermedia* Norway
MN130078 *Russula castanopsidis* Korea
MN130099 *Russula purpureogracilis* Thailand
MN130100 *Russula purpureogracilis* Thailand
UDB013265 *Russula* sp. Papua New Guinea
KU886598 *Russula mansehraensis* Pakistan
KR082870 *Russula maculata* China
KX441192 *Russula* sp. China
KX441179 *Russula* sp. China
JN129407 *Russula* sp. China
KU928159 *Russula nympharum* France
KU928157 *Russula nympharum* France
DQ422015 *Russula* cf. *maculata**
KU928156 *Russula maculata* Germany
KU928151 *Russula maculata* Estonia
KU928150 *Russula maculata* Slovakia
UDB016077 *Russula maculata* Estonia
UDB002498 *Russula maculata* Denmark
KU886597 *Russula maculata* Denmark
KU928154 *Russula maculata* Germany
UDB002497 *Russula maculata* Denmark
KU928152 *Russula maculata* Germany
MK105630 *Russula candida* Germany
MK105631 *Russula candida* Spain
MN130060 *Russula* sp. South Korea
KU886591 *Russula cuprea* Sweden
KU886592 *Russula cuprea* Germany
KU928148 Russula juniperina Germany
*KU928149 Russula juniperina**
KU886596 Russula juniperina Spain
UDB011303 *Russula font-queri* Estonia
KU949378 *Russula font-queri**
UDB011314 *Russula font-queri* Estonia
UDB011116 *Russula aurantioflammans* Finland
KU928167 *Russula aurantioflammans**
UDB015060 *Russula aurantioflammans* Sweden
KY693660 *Russula puellula* Spain
AY061710 *Russula puellula**
UDB015987 *Russula nitida* Estonia
UDB011221 *Russula nitida* Estonia
MN130082 *Russula* sp. Mexico
MN130083 *Russula* sp. Mexico
UDB002543 *Russula xerampelina* Sweden
JQ888204 *Russula xerampelina* Scotland
KU205278 *Russula xerampelina* Sweden
MN130095 *Russula* sp. Mexico
MN130094 *Russula* sp. Mexico
MN130101 *Russula* sp. Mexico
MN130093 *Russula* sp. Mexico
KU928140 *Russula decipiens* Slovakia
KU928139 *Russula decipiens* Germany
KU928138 *Russula decipiens* Spain
UDB011056 *Russula vinososordida* Finland
UDB011097 *Russula vinososordida* Denmark
KY509511 *Russula subtilis* USA
KY509512 *Russula subtilis* USA
KY509504 *Russula subtilis USA*
KY509508 *Russula subtilis USA*
KT933992 *Russula decolorans* Germany
UDB001643 *Russula vinosa* UK
UDB011204 *Russula vinosa* Estonia
UDB000902 *Russula vinosa* Sweden
KU928137 *Russula badia* France
KU928169 *Russula badia**
MN130108 *Russula seperina* Slovakia
MN130097 *Russula olivaceohimalayensis* India
MN130098 *Russula olivaceohimalayensis* India
KU928165 *Russula velernosa* Belgium
KU928166 *Russula velernosa* Slovakia
KU928164 *Russula velernosa* Slovakia
KU928142 *Russula firmula* Germany
KU886593 *Russula firmula* Poland
KU886599 *Russula rubra* Germany
KU928161 *Russula rubra* Sweden
KU928162 *Russula rutila* Slovakia
KU886600 *Russula rutila* Slovakia
KU928163 *Russula rutila* Germany
KT933999 *Russula sardonia* Germany
JQ888196 *Russula emetica* Scotland
Outgroup
0.02

Fig. 1. Molecular analysis of *Russula rubricolor* based on ITS sequences. The evolutionary history was inferred by the Maximum likelihood. The sequences generated during this study are shown with bold text. Red represents the sequence from type collection. Scale bar indicates the number of nucleotide substitutions per site. Asterisk (*) represents unspecified sampling area. Green dot represents *R. globispora* complex and red dot represents *R.* subsect. *Maculatinae*.

its independent position in *R.* subsect. *Maculatinae*. The clades recovered in the phylogenetic analysis correspond to those in Adamčík & al. (2016, 2019) and Li & al. (2018).

Taxonomy

Russula rubricolor Jabeen, Naseer & Khalid, **sp. nov.** Figs 2–4

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> Differs from *Russula heilongjiangensis* by its larger pileus and larger basidiospores with isolated to fused warts.
>
> Type: Pakistan, Khyber Pakhtunkhwa province, Hazara division Abbottabad district, Shimla Hill, 1500 m a.s.l., on soil under *Cedrus deodara* (Roxb. ex D. Don) G. Don, 21 Aug, 2014, Sana Jabeen & Abdul Nasir Khalid SJ105 (**Holotype** LAH35071; GenBank MK142796).
>
> Etymology: The specific epithet *rubricolor* refers to red color of the pileus.

Pileus 5–8.2 cm broad, convex to plane, infundibuliform in age, centre depressed; surface dry, smooth; margin striate, incurved; yellowish red (7YR 3.6/6) when young, becoming bright red (3.7YR 1.6/4.8) to blackish red (2.5YR 0.9/1.8) when mature. Lamellae 0.2–0.3 cm broad, adnexed, light yellow (6.5Y 5.8/3.2), subdistant to close, regular, entire; lamellulae absent. Stipe 4.2–6 cm long, 1.5–2 cm diam., central, cylindrical, slightly broader towards the base, white (8.8GY 8.9/0.7), smooth to finely wrinkled, fibrillose. Odor acrid. Taste not observed. Spore print not obtained.

Basidiospores [100/5/3] (9.7–)10.5–10.7(–11.5) × (6.7–)7.6–8.6(–9) µm, Q = (1.1–)1.2–1.3(–1.6), avQ = 1.2, subglobose to broadly ellipsoid (rarely ellipsoid), hyaline to pale yellow in 5% KOH; ornamentation amyloid, composed of prominent warts (0.3–0.6 µm high) that are isolated to fused in long chains; apiculus prominent. Basidia (38.3–)41.4–43.1(–44.6) × (10.1–)12.6–14.7(–16.5) µm, clavate to subclavate, tetrasterigmate, contents present. Cheilocystidia (44–)54.6–90.2(–131.3) × (10.2–)13.4–18.3(–22.5) µm, fusiform, bear dense crystalline contents, hyaline in KOH, pink in Congo red, grey in sulphovanillin. Pleurocystidia (89.4–)91.4–106.7(–110.2) × (9.8–)11.6–13.2(–14.7) µm, narrowly fusiform, hyaline in KOH, pink in Congo red, grey in sulphovanillin. Pileipellis 80.8 µm thick trichoderm; hyphae (2–)2.1–2.3(–2.6) µm wide, branched, septate. Pileocystidia (2.3–)2.4–2.7(–2.8) µm wide, single-celled, clavate to cylindrical, with rounded apex, non-incrusted, hyaline in 5% KOH, grey in sulphovanillin; dense at the center becoming sparse towards the margin. Stipitipellis a cutis; hyphae (1.3–)1.6–2.4(–3.2) µm wide, filamentous, branched, septate, hyaline in 5% KOH.

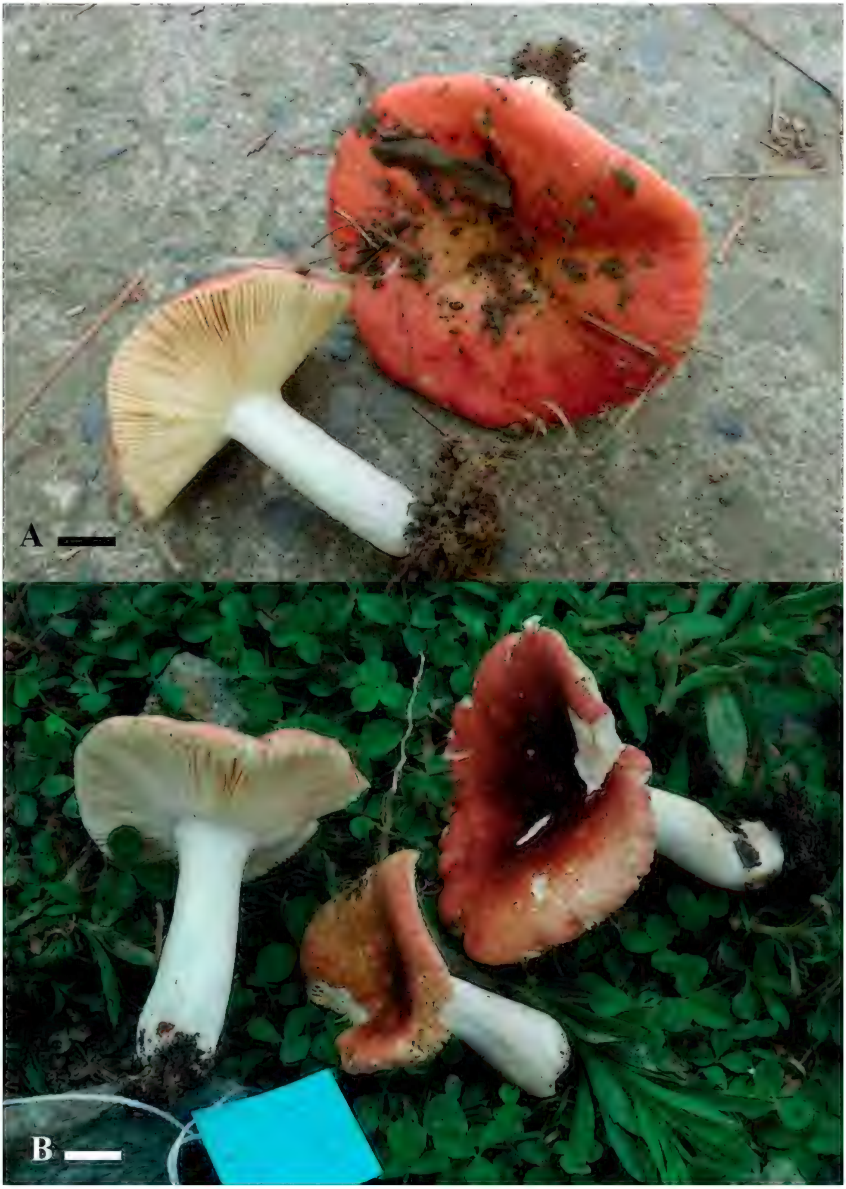

Fig. 2. *Russula rubricolor.* A. LAH35071 (holotype); B. LAH35248. Scale bars = 1 cm.

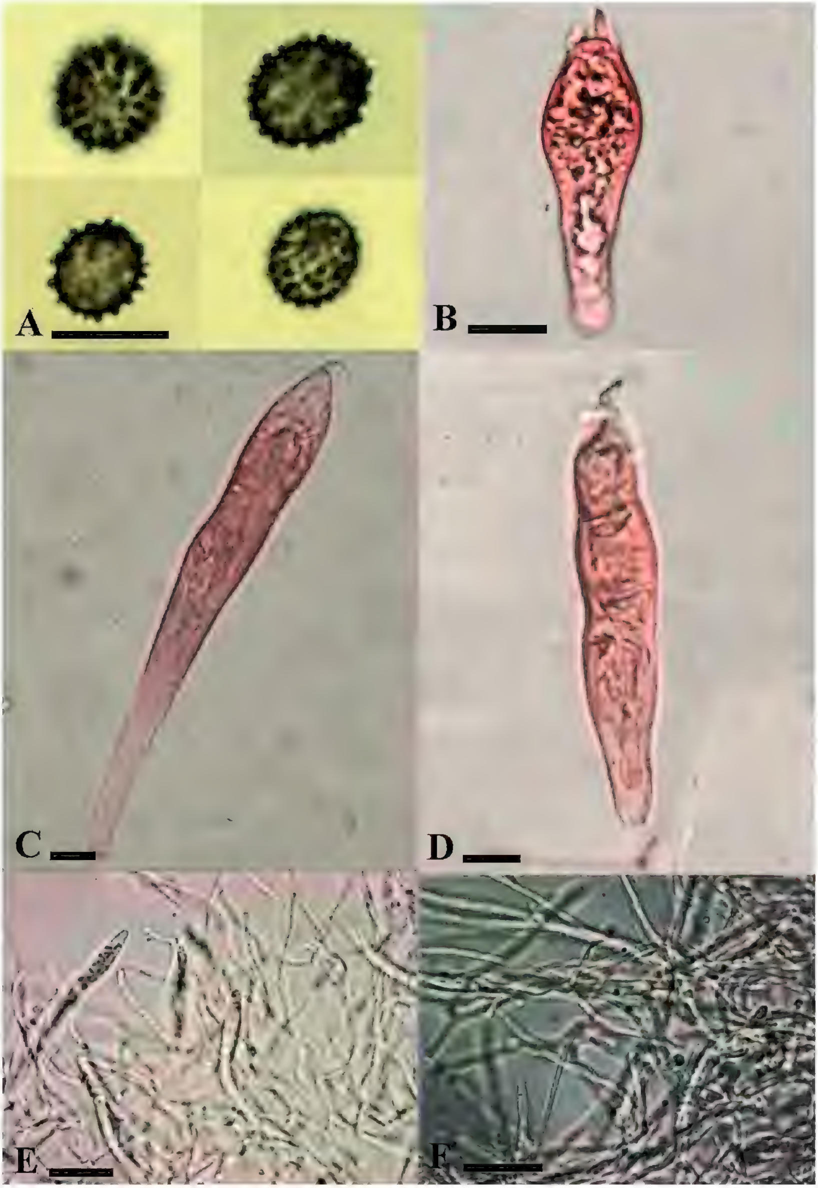

FIG. 3. *Russula rubricolor* (holotype, LAH35071). A. Basidiospores from different angles; B. Basidia; C. Cheilocystidia; D. Pleurocystidia; E. Pileocystidia in pileipellis; F. Stipitipellis. (A in Melzer's reagent; B–D in Congo red; E, F in KOH). Scale bars = 10 µm.

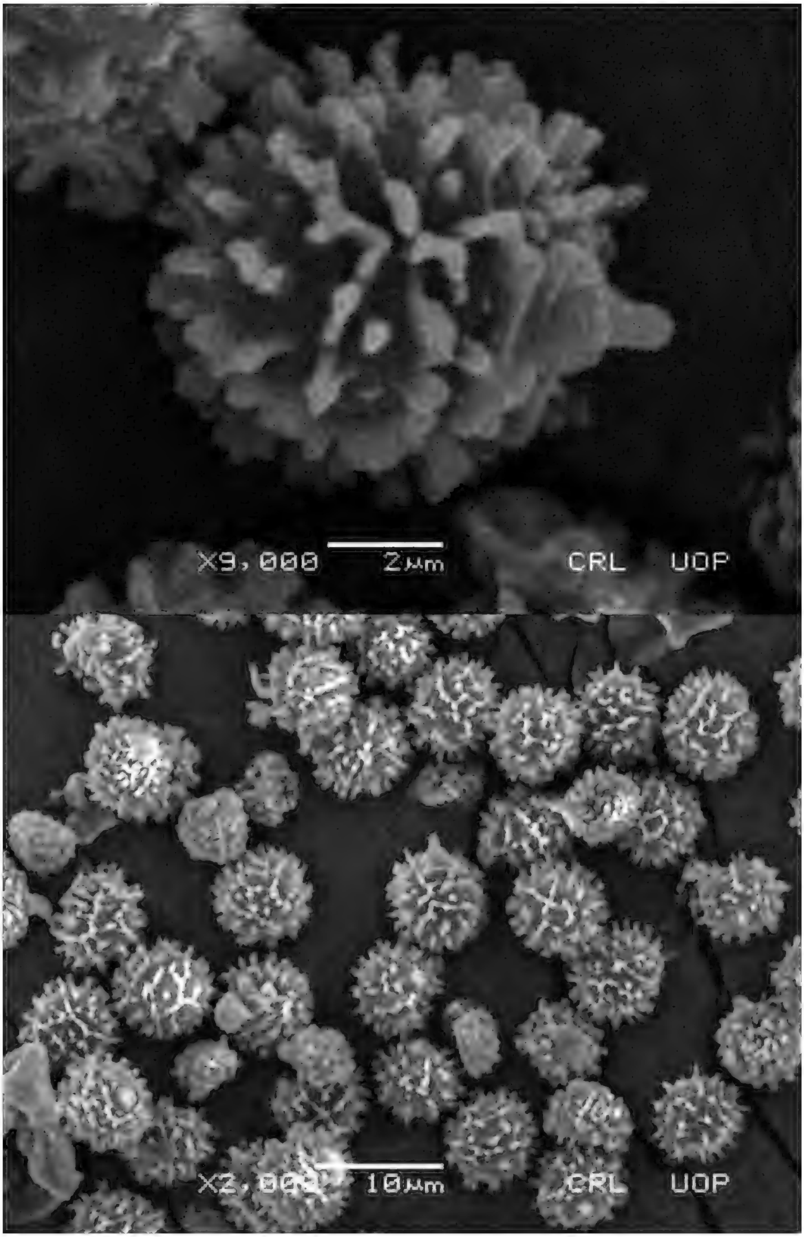

Fig. 4. *Russula rubricolor* (LAH35248). Basidiospores (SEM).

Additional specimens examined: **PAKISTAN. Khyber Pakhtunkhwa, Malakand division**, Swat district, Mashkun, 2500 m a.s.l., on soil under *Cedrus deodara*, 6 August 2014, Sana Jabeen & Abdul Nasir Khalid SJ106 (LAH35070; GenBank MK142795); Toa, 2800 m a.s.l, on soil under *Quercus* sp., 15 July 2015, Arooj Naseer & Abdul Nasir Khalid, ASTB47 (LAH35248; GenBank MK142797, MK142798).

Habitat & distribution—Himalayan moist *Pinaceae*-dominated forests (including *Pinus roxburghii*, *P. wallichiana*, and few *Cedrus deodara*) and dry oak-dominated temperate forests (including *Quercus oblongata*). Currently known only from Pakistan's Khyber Pakhtunkhwa province.

Discussion

Species in *Russula* subsect. *Maculatinae* are characterized by the combination of a red pileus and pileocystidia turning greyish in sulphovanillin (Romagnesi 1967, 1985). The newly described *R. rubricolor* is distinguished primarily by its yellowish hymenium, convex to infundibuliform, yellowish red to bright red or blackish red pileus that is striate towards the edge and which becomes blackish red with age. It is also characterized by large broadly ellipsoid basidiospores covered with isolated to fused warts. Both morphological and phylogenetic analyses confirm the independence and position of *R. rubricolor* in *R.* subsect. *Maculatinae* (Fig. 1), as defined by Adamčík & al. (2016, 2019).

Russula rubricolor clusters in a clade with some unidentified *Russula* species and three closely related species that comprise the *R. globispora* complex. *Russula dryadicola* (described from central European alpine habitats) differs from *R. rubricolor* by its yellow and purple pileus lacking bright red tinges and its medium-sized (4–6 cm) pileus (Sarnari 1998), while the European *R. globispora* is distinguished by its variously colored pilei and longer (41–53 µm) basidia (Sarnari 1998, Adamčík & Jančovičová 2013). *Russula heilongjiangensis* from northeastern China differs from *R. rubricolor* in its smaller (2.8–5.2 cm) pileus (Li & al. 2018).

Another Pakistani species, *Russula abbottabadensis* Saba & Adamčík, was described from the same habitat as *R. rubricolor*. Macroscopically similar to *R. rubricolor, R. abbottabadensis* can be separated by its smooth (not striate) pileal margins, larger (9.9–10.6 × 7.6–8.3 + 3–6 µm ornamentation) basidiospores, and 1–2-celled pileocystidia (Caboň & al. 2019). *Russula abbottabadensis* also clustered in the *Russula* crown clade, but very distant from *R. rubricolor*.-

Russula sharmae K. Das & al., an Indian taxon described based solely on morphological features, can be separated from *R. rubricolor* by its viscid to glutinous pileus with a strongly sulcate margin, lamellae that fork near the pileus edge, smaller (6.8–7.9 × 6.0–7.0 µm + 1 µm ornamentation) basidiospores, and fusiform to subclavate pleurocystidia with rounded apices (Das & al. 2013).

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